AD_____

GRANT NO: DAMD17-94-J-4410

TITLE: Int-3 Oncogene in Normal and Neoplastic Breast Development

PRINCIPAL INVESTIGATOR(S): Doctor Jan Kitajewski

CONTRACTING ORGANIZATION: Columbia University

New York, New York 10032

REPORT DATE: September 1995

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for public release;
distribution unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	September 1995	Annual 1 Sep 94 -31 Aug 95

4. TITLE AND SUBTITLE
Int-3 Oncogene in Normal and Neoplastic Breast Development

5. FUNDING NUMBERS
DAMD17-94-J-4410

6. AUTHOR(S)
Dr. Jan Kitajewski

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Columbia University
New York, New York 10032

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 words)*

Mutational activation of the murine *int-3* oncogene can contribute to mammary gland tumorigenesis. Evidence suggests that int-3 regulates the morphogenesis and functional differentiation of the mouse mammary gland. The proposed research investigates the role of int-3 proteins in the normal physiology of breast development and the biochemical properties critical for int-3 transforming activity.

cDNAs encoding the truncated *int-3* oncogene were cloned. A full length cDNA of approximately 7.3 kilobases was subsequently cloned and has been sequenced. The deduced amino acid sequence verifies that *int-3* is a *Notch/lin-12* family member. Structurally, the int-3 protein contains approximately 20 EGF-like repeats, 3 Notch/lin-12 repeats, a transmembrane domain, and an intracellular domain containing six ankyrin repeats. Analysis of *int-3* mRNA shows expression in several adult tissues and during mouse embryonic development. Cell lines have been generated that express an epitope-tagged int-3 oncoprotein, which was detected by Western blot and immunofluorescence analysis. Activated int-3 oncoproteins were found primarily in the nucleus and putative nuclear localization signals have been identified. Finally, bacterially produced int-3 proteins have been purified and used to generate rabbit polyclonal serum.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Int-3, mammary oncogene, transformation	19
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

GENERAL INSTRUCTIONS FOR COMPLETING SF 298

The Report Documentation Page (RDP) is used in announcing and cataloging reports. It is important that this information be consistent with the rest of the report, particularly the cover and title page. Instructions for filling in each block of the form follow. It is important to ***stay within the lines*** to meet ***optical scanning requirements***.

Block 1. Agency Use Only *(Leave blank)*.

Block 2. Report Date. Full publication date including day, month, and year, if available (e.g. 1 Jan 88). Must cite at least the year.

Block 3. Type of Report and Dates Covered. State whether report is interim, final, etc. If applicable, enter inclusive report dates (e.g. 10 Jun 87 - 30 Jun 88).

Block 4. Title and Subtitle. A title is taken from the part of the report that provides the most meaningful and complete information. When a report is prepared in more than one volume, repeat the primary title, add volume number, and include subtitle for the specific volume. On classified documents enter the title classification in parentheses.

Block 5. Funding Numbers. To include contract and grant numbers; may include program element number(s), project number(s), task number(s), and work unit number(s). Use the following labels:

C	Contract	**PR**	Project
G	Grant	**TA**	Task
PE	Program Element	**WU**	Work Unit Accession No.

Block 6. Author(s). Name(s) of person(s) responsible for writing the report, performing the research, or credited with the content of the report. If editor or compiler, this should follow the name(s).

Block 7. Performing Organization Name(s) and Address(es). Self-explanatory.

Block 8. Performing Organization Report Number. Enter the unique alphanumeric report number(s) assigned by the organization performing the report.

Block 9. Sponsoring/Monitoring Agency Name(s) and Address(es). Self-explanatory.

Block 10. Sponsoring/Monitoring Agency Report Number. *(If known)*

Block 11. Supplementary Notes. Enter information not included elsewhere such as: Prepared in cooperation with ...; Trans. of ...; To be published in .... When a report is revised, include a statement whether the new report supersedes or supplements the older report.

Block 12a. Distribution/Availability Statement. Denotes public availability or limitations. Cite any availability to the public. Enter additional limitations or special markings in all capitals (e.g. NOFORN, REL, ITAR).

- **DOD** - See DoDD 5230.24, "Distribution Statements on Technical Documents."
- **DOE** - See authorities.
- **NASA** - See Handbook NHB 2200.2.
- **NTIS** - Leave blank.

Block 12b. Distribution Code.

- **DOD** - Leave blank.
- **DOE** - Enter DOE distribution categories from the Standard Distribution for Unclassified Scientific and Technical Reports.
- **NASA** - Leave blank.
- **NTIS** - Leave blank.

Block 13. Abstract. Include a brief *(Maximum 200 words)* factual summary of the most significant information contained in the report.

Block 14. Subject Terms. Keywords or phrases identifying major subjects in the report.

Block 15. Number of Pages. Enter the total number of pages.

Block 16. Price Code. Enter appropriate price code *(NTIS only)*.

Blocks 17. - 19. Security Classifications. Self-explanatory. Enter U.S. Security Classification in accordance with U.S. Security Regulations (i.e., UNCLASSIFIED). If form contains classified information, stamp classification on the top and bottom of the page.

Block 20. Limitation of Abstract. This block must be completed to assign a limitation to the abstract. Enter either UL (unlimited) or SAR (same as report). An entry in this block is necessary if the abstract is to be limited. If blank, the abstract is assumed to be unlimited.

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the US Army.

N/A Where copyrighted material is quoted, permission has been obtained to use such material.

N/A Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

✓ Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

✓ In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Resources, National Research Council (NIH Publication No. 86-23, Revised 1985).

N/A For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

✓ In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

✓ In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

✓ In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

9/27/95

PI - Signature Date

INT-3 ONCOGENE IN NORMAL AND NEOPLASTIC BREAST DEVELOPMENT
Annual Report 1995

TABLE OF CONTENTS

INTRODUCTION	pages	2-5
BODY	pages	6-11
CONCLUSIONS	pages	12
REFERENCES	pages	13-16

INTRODUCTION

I. NATURE OF THE PROBLEM

In the past several years, it has been shown that oncogenes contribute to the pathobiology of breast cancer. Mutational activation of the *int-3* oncogene, has been shown to contribute to experimental mammary gland tumorigenesis in mouse. Several human orthologues of the *int-3* gene have been implicated in human cancers. There is strong evidence that the int-3 protein regulates the cell fate decisions required for the morphogenesis and functional differentiation of the mouse mammary gland. Despite this evidence, work on the role of the *int-3* gene in breast cancer is still in its infancy. Extensive studies on other int-3 family members (lin-12/Notch) in organisms more tractable to genetic analysis such as Drosophila and C. elegans, demonstrates the evolutionary conservation of these proteins and their fundamental importance in cell fate decisions.

The proposed research will investigate the role of the int-3 protein in the normal physiology of breast development and study the biochemical properties that are important for int-3 transforming activity. This information will broaden our understanding about the events which control normal mammary gland development and how alterations in those events can lead to neoplastic growth of the mammary gland.

II. BACKGROUND

Mouse mammary tumor virus induces breast cancer in mouse by insertional mutagenesis. In tumors, viral integration can result in activation of the *int-3* gene by promoter insertion and results in expression of a truncated *int-3* gene product (2.3 kb RNA) (1). The nucleotide sequence of this truncated cDNA revealed homology with the Notch/lin-12 gene family (2). However the full length cDNA of the *int-3* gene has not been cloned. Several lines of evidence confirm a role for int-3 in mammary tumorigenesis. Transfection of a recombinant *int-3* genomic DNA fragment, encoding the truncated oncoprotein, into the HC11 mouse mammary epithelial cell line induces anchorage-independent growth in soft agar (2). Expression of this same genomic fragment *in vivo* as a transgene in a transgenic mouse strain is associated with arrest of normal mammary gland development and impaired differentiation (3), intraductal hyperplasia of mammary epithelium, and a high incidence of focal mammary tumors (adenocarcinomas) (4). It has also been reported that the normal *int-3* gene is endogenously expressed in the mouse mammary gland (5).

Int-3 is related to the Notch/lin-12 family of proteins. The Notch/lin-12 protein family currently consist of eleven members, Notch (Drosophila) (6), lin-12 and glp-1 (C. Elegans) (7, 8, 9), Xotch (Xenopus) (10), Notch 1, 2, 3 and int-3 (Mouse) (2, 11-15), Notch 1 and 2 (Rat) (16,17), NOTCH 1 and 2 (Human) (18, 19). These genes encode for transmembrane receptor proteins. The extracellular domain of Notch/lin-12 family members contains variable numbers of EGF (epidermal growth factor) like repeats and other cysteine rich repeats named lin-12/Notch repeats(26). The intracellular domain of all Notch/lin-12 family members contains several copies of a repeat sequence, named cdc10 or ankyrin repeat. The cdc10 repeats have recently been implicated as a protein-protein interaction domain. The intracellular domain of this family of proteins also contains a PEST sequence, a nuclear localization signal, and an opa repeat. PEST sequences are found in proteins which are rapidly degraded or may also represent potential phosphorylation sites. An opa repeat is a protein domain that is rich in glutamine and is commonly found in transactivating domains of transcription factors or transcription factor binding proteins (27). The Notch and lin-12 proteins are required for cell-cell interactions that play a pivotal role in cell-fate decisions. For instance, the mechanisms that control how a group of equivalent progenitor cells give rise to a group of cells each with their particular fate. The fundamental importance of these genes during development has been demonstrated by genetic analysis of lin-12, Notch and Xotch mutants (20-24). In the mouse, nul mutants of Notch 1 and 2 are lethal during embryonic development, although the exact cause for this premature death is not known (25 and unpublished data T. Gridley).

Genetic and molecular analysis have identified several proteins that participate in Notch signaling. Drosophila *Delta* (28) and *Serrate* (29) and C. elegans *Lag-2* (30)and *Apx-1* encode a family of structurally related ligands for the Drosophila Notch and C.elegans lin-12 and glp-1 receptors respectively. These ligands are transmembrane proteins, containing EGF-like domains and a cysteine rich DSL (Delta-Serrate-Lag-2) domain within the extracellular part of the protein. Recently, mouse homologs of these ligands have been cloned, *Jagged-1* (31) and *Dll-1* (32). These ligands have been demonstrated to regulate Notch receptor activity through cell-cell interactions. The products of three Drosophila genes, deltex, disheveled and suppressor of hairless (Su(H))have been shown to interact with the intracellular domain of Notch and may thus participate in the intracellular signaling pathway of Notch (33,34) Furthermore, genetic analysis has revealed similar phenotypes in certain Deltex and Notch mutants.

Deletion of the extracellular part of Notch, Xotch and lin-12 proteins results in a dominant gain of function mutation (20-22). The truncated gene product encoding for the intracellular part of the receptor exhibits constitutively activated protein function. The phenotype observed in this class

of mutants suggests that the truncated gene products delay cell determination and thereby increase the proportion of uncommitted stem cells, leading to a prolonged lifetime of the cell or to a greater number of descendants (20-22). By analogy to the function of other Notch/lin-12 family members in lower organisms, one can speculate that delay in differentiation and accumulation of pluripotent proliferative stem cells would result in a growth advantage, thereby increasing the probability for secondary oncogenic mutations. This model would propose that Notch proteins contribute to oncogenesis by stimulating stem cell growth and blocking differentiation.

Studies on the Notch protein in Drosophila, demonstrated that the intracellular part of the Notch protein is translocated to the nucleus when a truncated Notch protein (corresponding to the intracellular part of the protein) is expressed as a transgene in Drosophila embryos (20). Based on the hypermorphic effect of the deletion mutants, and on the presence of a nuclear translocation signal in the intracellular domain of the protein, a hypothetical model would be that ligand binding to the receptor would result in cleavage of the intracellular domain of the receptor and subsequent translocation to the nucleus, where it could interact with its substrate.

Notch/lin-12 gene family members have been implicated in human tumorigenesis. Alteration of NOTCH-1 (also named TAN-1) has been associated with a T lymphoblastic neoplasm (18). The mutation of the NOTCH-1 gene in T lymphoblastic lymphomas is caused by a translocation that results in expresion of a truncated gene product. TAN-1 mutations are analogous to the *int-3* activating mutations as a result of MMTV insertion, as well as to the dominant gain of function mutations of Notch, lin-12 and Xotch. Furthermore, human NOTCH-1 and NOTCH-2 (also named hN) were found to be overexpressed in human cervical carcinomas (19).

III. PURPOSE

The *overall goal* of the work proposed here is to understand in molecular detail the function of the int-3 protein in mammary epithelial cells and during mouse mammary gland development, with the *long term goal* of understanding the role of the *int-3* gene in mammary tumorigenesis.

IV. METHODS OF APPROACH

Our *general strategy* is to carry out an analysis of the normal int-3 protein and the oncogenic int-3 protein to determine how these proteins contribute to normal and neoplastic breast development by addressing the following *specific aims*:

Aim 1. *Compare the normal int-3 protein to activated int-3 oncoproteins.* The first step of this section will be to determine the primary sequence of the normal int-3 protein and evaluate whether the normal *int*-3 gene encodes a Notch-like transmembrane receptor. The second step will be to compare the biochemical properties, subcellular localization, and transforming activities of the normal int-3 protein with that of truncated int-3 oncoproteins. Our aim is to determine what structural alterations lead to oncogenic activation of int-3 and to evaluate whether activated int-3 proteins act at the cell surface and/or nucleus.

This aim has been divided into the following two *tasks*:

Task 1. *Characterize the full length int-3 cDNA* (Months 1-12).

We have cloned the truncated, oncogenic *int-3* gene and we have cloned and sequenced the full length *int-3* gene.

Task 2. *Study the production, processing, and subcellular localization of normal int-3 proteins and int-3 oncoproteins in mammary epithelial cells* (Months 3-18).

We have epitope-tagged int-3 proteins, we have generated cell lines expressing the truncated int-3 oncoprotein and we have evaluated the subcellular localization of int-3 oncoproteins.

Aim 2. *Analysis of int-3 expression pattern in the murine mammary gland.* The fact that expression of the *int*-3 oncogene in the mouse mammary gland has profound effects on the development of the gland suggests a role of the normal *int*-3 gene products in mammary gland development. We will investigate whether the *int*-3 gene is normally expressed in the mammary gland. More specifically, we will determine if *int*-3 expression is found during distinct stages of mammary gland development and in distinct cell types of the mouse mammary gland. Molecular and immunohistochemical techniques will be used to evaluate where and when in the normal mammary gland the *int*-3 gene is expressed.

We have made early progress on some tasks associated with this *specific aim*:

Task 5 (of proposal). *Analysis of the int-3 expression pattern in the murine mammary gland* (Months 12-48). We have initiated the generation of int-3 specific antibodies and defined the expression pattern of this gene in mouse embryos and adult tissues.

BODY

I. EXPERIMENTAL METHODS/RESULTS

Characterization of the full length *int-3* cDNA.

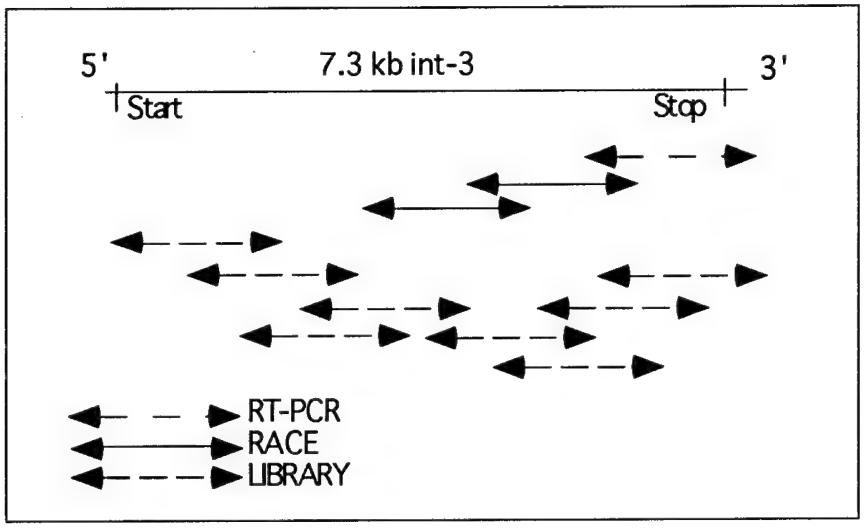
Int-3 has been classified in the lin-12/Notch protein family solely on the basis of its homology to the intracellular part of the lin-12/Notch family members. There has been no direct evidence that demonstrates that int-3 is a transmembrane protein. By cloning the entire full length *int-3* cDNA, we have confirmed that the *int-3* gene encodes for a transmembrane protein, homologous to the lin-12/Notch family of transmembrane proteins.

A. Cloning truncated *int-3*. We have PCR amplified a fragment of the *int-3* gene that encodes for the intracellular part of the protein. Oligonucleotide primers were designed based on the published sequence (2). Mouse adult testis cDNA was used as template DNA in the PCR reaction. Appropriate sized fragments resulted from the PCR reaction and internal primers were used to confirm the identity of these PCR fragments. We have cloned the PCR products into a phagemid (pBluescript). Sequencing of these cloned fragment, revealed almost exact nucleotide identity to the published sequence (2). Some sequence differences result in a sligtly different predicted amino acid sequence, for instance the coding frame predicts a transmembrane domain which was not reported in the published sequence of *int-3* (2).

B. Cloning of normal *int-3*. We have used the PCR based method of RACE (Rapid Amplification of cDNA Ends) (35,36) to clone sequences that are located 5' from the truncated *int-3* (the truncated *int-3* transcript is localized at the 3' end of the gene). To clone the *int-3* full length cDNA, we screened a mouse lung cDNA library. This choice of cDNA library was based on my findings of the mRNA expression analysis of *int-3* (task # 5). Initially, the probes that were used in this screening analysis were derived from the cloned truncated *int-3*, as well as from the clones obtained by RACE. Positive clones were purified and sequenced and used as probes in successive rounds of screening in order to obtain the full length cDNA of the *int-3* gene. The length of the *int-3* mRNA is approximately 7.3 kb based on our Northern blot analysis (task # 5). To date, I have cloned the 7.3 kb full length *int-3* cDNA (Fig 1). The cDNA contains a 400 bp 3' UTR (untranslated region) and a 500 bp 5' UTR, resulting in a 6.4 kb ORF (open reading frame). I have determined the initiating ATG based on Kozak rules, and based on its location 5' of a putative signal peptide domain. The *int-3* sequence has been analyzed for its homology with other lin-12/Notch family members, and has a high overall homology to the other known mouse *Notch* 1, 2

and 3 genes. It encodes for a transmembrane protein with a intracellular domain containing six ankyrin repeats, a transmembrane domain, and a extracellular domain containing three Notch/lin-12 repeats as well as up to more then twenty EGF-like repeats. We have also isolated *int-3* clones that are derived from differentially spliced *int-3* messages.

Fig 1: Schematic representation of *int-3* cloning



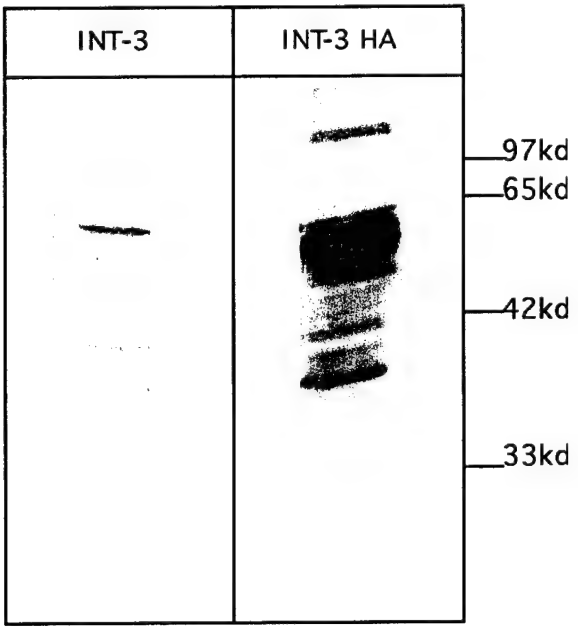
Determination of the biochemical properties of the int-3 protein.

The *int-3* gene was discovered in mouse mammary tumors, induced by MMTV infection. Insertional mutagenesis by MMTV results in expression of a truncated int-3 protein product that is able to transform mammary epithelium cells both in vivo and in vitro. We will analyze the biochemical characteristics of the int-3 protein using biochemical, biological and molecular strategies. We will compare the biochemical properties, subcellular localization and transforming activities of the normal int-3 protein with that of truncated int-3 oncoproteins.

A. Epitope tagging of truncated int-3. Since antibodies are not immediately available against the int-3 protein (task # 5), we have added an epitope to the coding region of truncated int-3(37). The epitope we have chosen to add to the int-3 protein is derived from the influenza HemAgglutinin (HA) protein and is recognized by a monoclonal antibody 12CA5 (38). *Int-3* cDNA was cloned into phagemid vectors that contain the sequence encoding the HA epitope situated downstream. Single strands were generated from the phagemid and used in a site directed mutagenesis protocol with an oligonucleotide designed to loop-out the sequence between the last

int-3 codon and the first HA codon, to create in frame a cDNA encoding the int-3/HA fusion protein. The HA epitope was fused to the carboxy terminus of the int-3 protein. This method allowed me to detect ectopically expressed int-3/HA fusion protein in transfection experiments, using anti HA antibodies in Immunoblot analysis. Cell lysates were prepared from int-3 expressing cell lines, fractionated by SDS gel preparation, and transferred to nitrocellulose. This membrane was blotted with anti HA antibodies and the truncated int-3 protein was detected by ECL (enhanced chemiluminescence) followed by autoradiography (Fig 2).

Fig 2: Western blot analysis on total cell lysates of transient transfected BOSC cells



B. Construction of truncated int-3 expressing cell lines. An *int-3/HA* fusion construct was subcloned into a murine leukemia virus (MLV) based vectors, denoted LNCX vectors (39). These vectors utilize the cytomegalovirus immediate early promoter/enhancer to drive expression of the *int-3* gene and the retroviral LTR to drive expression of the *neo* gene, which confers resistance to the drug G418. We have also generated several control retroviral vectors, such as vectors not containing *int-3*, vectors containing an unrelated HA tagged protein, and vectors containing *int-3* not HA tagged. This last control vector will allowed us to investigate whether HA tagging (at the carboxy terminus) can interfere with the transforming activity of int-3. In order to evaluate the biochemical properties and transforming potential of int-3, mammary epithelial cell lines programmed to express int-3 were generated by infection with retroviral expression vectors. High-titer helper free retroviral stocks were generated using the BOSC23 ecotropic virus packaging cell line (40), media was collected and used to infect mammary epithelial cell lines and fibroblast cell

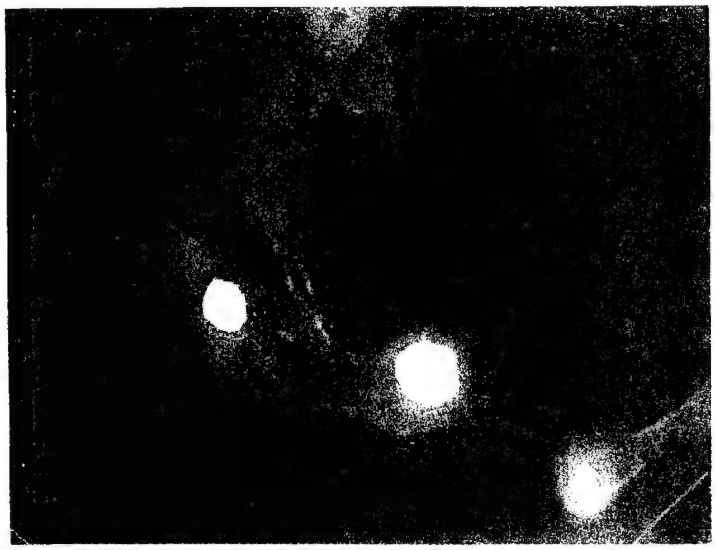
lines. The mammary epithelial cell lines that will be used, are C57MG(41), HC11(42), TAC-2 (43) and MCF7(44). HC11 will be used because it has been reported that this cell line can be transformed by truncated int-3 protein (2) In addition we will infect other established mammary epithelial as well as non-epithelial cell lines. Once these cell lines are established, the biochemical properties and transforming activity of int-3 will be investigated.

Determine the subcellular localization of int-3 proteins.

The biochemical properties of int-3 will be investigated once the full length cDNA has been expressed in mammary epithelial cells. Initially, we have determined the intracellular localization of truncated int-3.

From studies on the Notch protein in Drosophila, there is evidence that truncated Notch protein (corresponding to the intracellular part of the protein) is translocated to the nucleus when this protein is expressed as a transgene in Drosophila embryos. Because the subcellular localization of truncated int-3 might give a clue as to how wild type int-3 functions, we have examined the intracellular localization of truncated int-3 in transiently transfected cells (293T and HeLa cells). We have investigated the intracellular localization by indirect immunofluorescence (45) using the anti-HA antibodies, and have found nuclear localization of truncated int-3 (Fig 3). Several deletion mutants have been generated in order to determine wich part of the truncated int-3 protein is responsible for its nuclear translocation. These initial studies pointed out two domains that may contain a nuclear translcation signal, one N-term and one C-term of the ankyrin repeats. Interestingly, deletion of the ankyrin repeats alone did not affect the nuclear localization. Preliminary data suggest that a protein consisting solely of the int-3 ankyrin repeats resides in the cytoplasm.

Fig 3: Nuclear localization of int-3 in transiently overexpressed 293T cells



Analysis of the int-3 expression pattern.

We will analyze the expression pattern of the int-3 protein as well as the *int-3* mRNA. Such analysis will give further insights into the function of the int-3 protein. First, we will investigate whether int-3 is expressed in the mammary gland and whether this expression is developmentally regulated. Second, we will study the expression pattern of int-3 in different mouse tissues, to define tissue specific expression or ubiquitous expression of int-3.

A. Generating int-3 specific antibodies. To study the endogenous expression pattern of the int-3 protein (task # 5), polyclonal antibodies against GST-int-3 fusion proteins are being generated. The intracellular part of the int-3 protein was fused in frame to GST and purified over a glutathionine column. Purified fusion proteins have been injected into two rabbits. The specificity of the polyclonal antibodies will be analyzed as soon as bleeds are available. We expect to obtain antiserum as soon as 10/95.

B. Expression analysis of int-3. Int-3 protein expression will be studied once the int-3 specific antibodies (see task # 5) for Western blot analysis are available. Immuno-histochemistry using those int-3 specific antibodies will be used to determine the specific localization of int-3 protein in tissue section and define the specifically the cell types expressing int-3 protein. *Int-3* mRNA expression has been studied by Northern blot analysis using probes derived from the 3' UTR of *int-3* (see task # 1). The *int-3* gene encodes for a 7.3kb transcript that is expressed in lung, heart, and kidney in adult tissues. Several shorter *int-3* transcripts were observed in adult testis, and appear to consist of alternatively spliced transcripts. The *int-3* transcript is expressed at all stages of mouse development (day 6.5 to 15.5). In situ hybridization (using the same probe as in the Northern blot analysis) was performed to determine the source of *int-3* expression during mouse development, and revealed endothelial specific expression. In situ hybridization on adult lung tissue is currently performed to determine the cell type in lung that expresses *int-3*.

II. GOALS OF THE RESEARCH

The goal of the proposed research is to investigate the role of the int-3 protein in the normal physiology of breast development and study the biochemical properties that are important for int-3 transforming activity. The data presented in this annual report represents reults of experiments outlined in *specific aim* 1 and *specific aim* 3 of the research proposal. We have completed all of *task* 1 of *specfic aim* 1(Months 1-12) by cloning both the truncated int-3 oncogene and a full length, 7.3 kb cDNA encoding normal int-3. We have completed approximately half of *task* 2 of *specific aim* 1 (Months 3-18) by epitope tagging int-3 proteins, by generating retroviral vectors for expressing int-3 proteins and by defining the subcellular localization of the int-3 oncoprotein. In addition, we have made early progress towards analyzing the int-3 expression pattern in the murine mammary gland, *task* 5-*specific aim* 3 (Months 12-48) of research proposal (aim 2 of this annual report). We have already purified bacterially produced int-3 proteins, injected these proteins into rabbits, and are commencing to determine whether the rabbits are producing int-3 specific antibodies. We have also defined the embryonic expression pattern of *int-3* and defined adult tissues where *int-3* is expressed. We will next look specifically in the murine mammary gland for *int-3* expression.

CONCLUSIONS

In conclusion, we have cloned both the truncated, oncogenic *int-3* gene and have cloned a full length cDNA of approximately 7.3 kb which encodes the normal int-3 protein. The deduced amino acid sequence of the normal int-3 protein displays significant homology to other Notch family members. The extracellular domain is characterized by the presence of a putative signal peptide followed by a series of EGF-repeats and Lin12/Notch repeats. The truncated *int-3* gene has been cloned and epitope tagged. Cell lines have been generated that are programed to express the truncated int-3 protein. The truncated int-3 protein expressed in epithelial cells was detected by Western blot and immunofluorescence analysis. We have found that the truncated int-3 protein is localized to the nucleus, suggesting that signal transduction may be mediated by the nuclear transport of the intracellular part of the int-3 cell surface receptors. Now that the full length *int-3* gene has been cloned, these experiments will be expanded to biological activity and biochemical properties of the normal int-3 protein.

The expression analysis of the *int-3* mRNA was analyzed in adult tissues as well as during mouse development. The *int-3* transcript consists of a 7.3 kb mRNA species found in lung, heart, kidney, and mouse embryos. Embryonic expression appears to be restricted to endothelial tissue. Future work will focus specifically on the expression of int-3 in the murine mammary gland.

References

1. Gallahan, D. and Callahan, R. Mammary Tumorigenesis in Feral Mice: Identification of a New int Locus in Mouse Mammary Tumor Virus (Czech II)-induced Mammary Tumors. *J. Virol.* 61, 66-74 (1987)

2. Robbins, J., Blondel, B.J., Gallahan, D. and Callahan, R. Mouse Mammary Tumor Gene int-3: a Member of the Notch Gene Family Transforms Mammary Epithelial Cells. *J.Virol.* 66, 2594-2599 (1992)

3. Smith, G.H., Gallahan, D., Diella, F., Jhappan, C., Merlino, G. and Gallahan, R. Constitutive expression of a truncated int-3 gene in mouse mammary epithelium impairs differentiation and functional development. *Cell Growth & Diff.* 6, 563-577 (1995)

4. Jhappan, C., Gallahan, D., Stahle, C., Chu, E., Smith, G.H., Merlino, G. and Callahan, R. Expression of an activated Notch-related int-3 transgene interferes with cell differentiation and induces neoplastic transformation in mammary and salivary glands. *Genes Devel.* 6, 345-355 (1992)

5. Sarkar, N.H., Haga, S., Lehner, A.F., Zhao, W., Imai, S. and Moriwak, K. Insertionsal mutaion of int protooncogenes in the mammary tumors of a new strain of mice derived from the wild in China: Normal- and Tumor-tissue specific expression of *int-3* transcripts. *Virology* 203, 52-62 (1994)

6. Wharton, K.A., Johansen, K.M., Xu, T. and Artavanis-Tsakonis, S. Nucleotide Sequence from the Neurogenic Locus Notch Implies a Gene Product That Shares Homology with Proteins containing EGF-like repeats. *Cell* 43, 567-581 (1985)

7. Yochem, J., Weston, K. and Greenwald, I. The Caenorhabditis elegans lin-12 gene encodes a transmembrane protein with overall similarity to Drosophila Notch. *Nature* 335, 547-550 (1988)

8. Greenwald, I. lin-12, A Nematode Homeotic Gene, Is Homologues to a Set of Mammalian Proteins That Includes Epidermal Growth Factor. *Cell* 43, 583-590 (1985)

9. Yochem, J. and Greenwald, I. glp-1 and lin-12, Genes Implicated in Distinct Cell-Cell Interactions in C. elegans, Encode Similar Transmembrane Proteins. *Cell* 58, 553-563 (1989)

10. Coffman, C., Harris, W. and Kintner, C. Xotch, the Xenopus Homolog of Drosophila Notch. *Science* 249, 1438-1441 (1990)

11. Lardelli, M. and Lendahl, U. Motch A and Motch B-Two Mouse Notch Homologues Coexpressed in a Wide Variety of Tissues. *Exp.Cell Res.* 204, 364-372 (1993)

12. Del Amo, F., Smith, D.E., Swiatek, P.J., Gendron-Maguire, M., Greenspan, R.J., McMahon, A.P. and Gridley, T. Expression pattern of Motch, a mouse homolog of Drosophila Notch, suggests an important role in early postimplantation mouse development. *Development* 115, 737-744 (1992)

13. Del Amo, F., Gendron-Maguire, M., Swiatek, P.J., Jenkins, N.A., Copeland, N.G. and Gridley, T. Cloning, Analysis, and Chromosomal localization of Notch-1, a Mouse Homolog of Drosophila Notch. *Genomics* 15, 259-264 (1993)

14. Reaume, A.G., Conlon, R.A., Zirngibl, R., Yamaguchi, T.P. and Rossant J. Expression Analysis of a Notch Homologue in the Mouse Embryo. *J.Dev.Biol.*154, 377-387 (1992)

15. Lardelli, M., Dahlstrand, J. and Lendahl, U. The novel Notch homologue mouse *Notch* 3 lacks specific epidermal growth factor-repeats and is expressed in proliferating neuroepithelium. *Mech Devel* 46, 123-136 (1994)

16. Weinmaster, G., Roberts, V.J. and Lemke, G. A homolog of Drosophila Notch expressed during mammalian development. *Development* 113, 199-205 (1991)

17. Weinmaster, G., Roberts, V.J. and Lemke, G. Notch 2: a second mammalian Notch gene. *Development* 116, 931-941 (1992)

18. Ellisen, L.W., Bird, J., West, D.C., Soreng, A.L., Reynolds, T.C., Smith, S.D. and Sklar,J. TAN-1, the human homolog of the Drosophila Notch gene, is broken by Chromosomal Translocations in T Lymphoblastic Neoplasms. *Cell* 66, 649-661 (1991)

19. Zagouras, P., Stifani, S., Blaumueller, C.M., Carcangiu, M.L. and Artavanis-Tsakonis, S. Alterations in Notch signaling in neoplastic lesions of the human cervix. *Proc.Natl.Acad.Sci.* USA 92, 6414-6418 (1995)

20. Struhl, G., Fitzgerald, K. and Greenwald, I. Intrinsic Activity of the Lin-12 and Notch Intracellular Domains In Vivo. *Cell* 74, 331-345 (1993)

21. Coffman, C.R., Skoglund, P., Harris, W.A. and Kintner, C.R. Expression of an Extracellular deletion of Xotch Diverts Cell Fate in Xenopus Embryos. *Cell* 73, 659-671 (1993)

22. Fortini, M.E., Rebay, I., Caron, L.A. and Artavanis-Tsakonas S. An activated Notch receptor blockscell-fate commitment in the developing Drosophila eye. *Nature* 365, 555-557 (1993)

23. de Celis, J.F., Mari-Befa, M. and Garcia-Bellido, A. Cell-autonomous role of Notch, an epidermal growth factor homologue, in sensory organ differentiation in Drosophila. *Proc.Natl.Acad.Sci.* USA 88, 632-636 (1991)

24. Lieber, T., Kidd, S., Alcamo, E., Corbin, V. and Young, M.W. Antineurogenic phenotypes induced by truncated Notch proteins indicate a role in signal transduction and may point to a novel function for Notch in nuclei. *Genes Devel* 7, 1949-1965 (1993)

25. Swiatek, P.J., Lindsell, C.E., Franco del Amo, F., Weinmaster, G. and Gridley, T. Notch 1 is essential for postimplantation development in mice. *Genes Devel.* 8, 707-719 (1994)

26. Stifani, S., Blaumueller, C.M., Redhead, N.J., Hill, R.E. and Artavanis-Tsakonas, S. Human homologs of a Drosophila Enhancer of Split gene product define a novel family of nuclear proteins. *Nature* Genetics 2, 119-127 (1992)

27. Wharton, K.A., Yedvobnick, B., Finnerty,V.G. and Artavanis-Tsakonas, S. Opa: a novel family of transcribed repeats shared by the Notch locus and other developmentally regulated loci in Drosophilla melanogaster. *Cell* 40, 55-62 (1985)

28. Kooh, P.J., Fehon, R.G. and Muskavitch, A.T. Implications of dynamic patterns of Delta and Notch expression for cellular interactions during *Drosophila* development. *Development* 117, 493-507 (1993)

29. Fleming, R.J., Scottgale, T.N., Diederich, R.J. and Artavanis-Tsakonis, S. The gene *Serrate* encodes a putative EGF-like transmembrane protein essential for proper ectodermal development in *Drosophila melanogaster. Genes Devel* 4, 2188-2201 (1990)

30. Henderson, S.T., Gao, D., Lambie, E.J. and Kimble, J. *lag-2* may encode a signaling ligand for the GLP-1 and LIN-12 receptors of *C. elegans. Development* 120, 2913-2924 (1994)

31. Lindsell, C.E., Shawber, C.J., Boulter, J. and Weinmaster, G. Jagged: A mammalian ligand that activates Notch 1. *Cell* 80, 909-917 (1995)

32. Bettenhausen, B., Hrabe de Angelis, M., Simon, D., Guenet, J-L. and Gossler, A. Transient and restricted expression during mouse embryogenesis of *DLL 1,* a murine gene closely related to Drosophila Delta. *Development* 121, 2407-2418 (1995)

33. Diederich, R.J., Matsuno, K., Hing, H. and Artavanis-Tsakonis, S. Cytosolic interaction between deltex and Notch ankyrin repeats implicates deltex in the Notch signaling pathway. *Development* 120, 473-481 (1994)

34. Matsuno, K., Diederich, R.J., Masahiro, J.G., Blaumueller, C.M. and Artavanis-Tsakonis, S. Deltex acts as a positive regulator of Notch signaling through interactions with the Notch ankyrin repeats. *Development* 121, 2633-2644 (1995)

35. Ohara, O., Dorit, R.L. and Gilbert, W. One-sided polymerase chain reaction: the amplification of cDNA. *Proc.Natl.Acad.Sci.USA* 86, 5673-5677 (1989)

36. Apte, A.N. and Siebert, P.D. Anchor-ligated cDNA libraries: a technique for generating a cDNA library for the immediate cloning of the 5' ends of mRNAs. *Bio. Techniques* 15, 890-893 (1993)

37. Kolodziej, P.A. and Young, R.A. Epitope tagging and protein surveillance. *Methods Enzymol.* 194, 508-519 (1991)

38. Wilson, I.A., Niman, H.L., Houghten, R.A., Cherenson, A.R., Connolly, M.L. and Lerner, R.A. The structure of an antigenic determinant of a protein. *Cell* 37, 767-778 (1984)

39. Miller, A.D. and Rosman, G.J. Improved retroviral vectors for gene transfer and expression. *Bio. Techniques* 7, 980-990 (1989)

40. Pear, W.S., Nolan, G.P., Scott, M.L. and Baltimore, D. Production of high titer helper-free retrovirus by transient transfection. *Proc.Natl.Acad.Sci.* USA 90, 8392-8396 (1993)

41. Vaidya, A.B., Lasfargues, E.Y., Sheffield, J.B. and Coutinho, W.G. Murine mammary tumor virus (MMuTV) infection of an epithelial cell line established from C57BL/6 mouse mammary glands. *Virology* 90, 12-22 (1978)

42. Danielson, K.G., Oborn, C.J., Durban, E.M. and Medina, D. Mouse mammary epithelial cell line exhibiting normal morphogenesis in vivo and functional differentiation in vitro. *Proc.Natl.Acad.Sci.* USA 81, 3756-3760 (1984)

43. Soriano, J.V., Pepper, M.S., Nakamuro, T., Orci, L. and Montesano, R. Hepatocyte growth factor stimulates extensive development of branching duct-like structures by cloned mammary gland epithelial cells. *J Cell Sci.* 108, 413-430 (1995)

44. Soule, H.D., Vazquez, J., Long, A., Albert, S. and Brennan, M. A human breast cancer line from a pleural effusion derived from breast carcinoma. *J. Natl. cancer Inst.* 51, 1409-1413 (1973)

45. Osborn, M. and Weber, K. Immunofluorescence and immunocytochemical procedures with affinity purified antibodies: Tubulin containing structures. *Methods Cell Biol.* 24, 97-132 (1982)